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CONFLICTING EXPRESSION PATTERN OF MIR-135 TARGET GENES DURING C2C12 DIFFERENTIATION

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ABSTRACT: In this study, we aimed to evaluate miR-135 candidate target genes during myoblast to myocyte differentiation process. Using a bioinformatics approach, ADIPOR2, STXBP4 and VAMP2 were selected as potential target of miR-135.Cell differentiation was induced by 3% horse serum. The expression level of genes were determined by qRT-PCR during differentiation process. The data about genes expression pattern during myoblast into myocyte differentiation demonstrated statistically significant down regulation of ROCK1 , and STXBP4 while ADIPOR2 and VAMP2 had upward expression level during the process. Seemingly, ADIPOR2 and VAMP2 are as potential regulators of myogenesis and involve in skeletal muscle development through mTOR/PI3K pathway , maintenance and development of muscle cells. Decreased expression of STXBP4 and ROCK1 accompanied with overexpression of miR-135 (as its possible targets) could be involved in the control the vesicle translocation and membrane fusion which is required for development of cells during myogenesis. all at once, it seems that, miR-135 could be involved in myogenesis via down regulation of STXBP4 and ROCK-1(as negative regulators) except for ADIPOR2 and VAMP2 which their over expression is essential for muscle cell differentiation.

Keywords: miRNA, Gene, C2C12 Differentiation.

INTRODUCTION

The process of myogenesis is extremely complex and requires a specific organization of signaling molecules, that regulates specific transcription, to activate particular gene and miRNAs expression (1,2).The fate of myogenic precursor , Myoblasts, is first determined by transcription factors, Pax3/Pax7, followed by regulation of myogenic differentiation (MyoD), and myogenic regulatory factors (MRFs) (3). MyoD is considered as a marker of terminal commitment to muscle fate.

The muscle differentiation is controlled by numerous signaling pathways such as PI3K/Akt/mTOR pathway (4). The PI3K/Akt/mTOR pathway promotes protein synthesis and,is essential for myotube formation (5-7).

Recently, microRNAs, a class of evolutionarily conserved and small non-coding RNAs (8), have emerged as novel and essential regulators of myogenesis (9). microRNAs can promote differentiation by repressing negative regulators of transcriptional activity or suppress it by repressing positive regulators. Several miRNAs have been demonstrated to regulate myogenesis. Base on our previous study, miR-135 was upregulated more than 70-fold in differentiated cells (10). Seemingly, miR-135 participates in myocyte formation process through targeting unknown components. The aim of this study is to measure expression changes of bioinformatically selected miR-135 target genes to better understand the role of miR-135 in molecular signaling of muscle differentiation process. In our survey, we have used C2C12 myoblast cells as widely used model for skeletal muscle cell development and have identified expression level of miR-135 target genes during C2C12 differentiation process.

MATERIALS AND METHODS

Cell Culture:

In our study, C2C12 myoblasts line were obtained from cell bank (Stem Cells Technology Research Center ,Tehran, Iran). These cells were cultured in growth medium (GM, Dulbecco's Modified Eagle Medium (Gibco) containing 10% fetal bovin serum (Gibco)) 24 hours before induced to differentiation, at 37 °C, 5% CO2. When cell density reached 70%, they were digested with 0.25% trypsin (Gibco), and then seeded into culture dishes. When inducing C2C12 myoblasts to differentiate, cell density must reach >90% prior to changing GM to differentiation medium (DM, Dulbecco's Modified Eagle Medium supplemented with 3% horse serum (Gibco)). The cells incubated with DM for other 72 hours to be differentiated. The control cell line (undifferentiated C2C12 line) were just cultured in growth medium for the same time. All cell culture were performed at least in triplicate.

Bioinformatics gene prediction:

Using Target Scan 6.2, miRWalk, we generated a list of candidate miR-135 target genes, containing a seed site for this miRNAs(11,12). We purely chose target genes for miRNA which had primarily predicted with at least two of the algorithms.

RNA isolation and qReal-Time PCR:

Cells were lysed and total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's instructions. The total RNA was processed into single strand cDNA using a reverse transcription kit (Fermentas) with random hexamer for genes. Real-time quantitative PCR was performed in triplicate using Rotor-gene Q real-time analyzer (Corbett, Sydney, Australia). Each real-time PCR system contained SYBR Premix Dimer EraserTM (TaKaRa), followed by melting curve analysis to confirm PCR specificity. The threshold cycle average was used for data analysis by Rotor-gene Q software (Corbett, Sydney, Australia). Genes and related specific primers are illustrated in Table 1. Gene expressions were normalized against the expression of β-actin and all reactions were run in triplicate. The $2^{-\Delta\Delta Ct}$ algorithm was employed to estimate the relative expression level of each gene.

Comparison of real time RT-PCR result by a highthroughput method data

We next asked whether the candidate gene expression levels obtained by real time-PCR are comparable with other methods. To this end, the results of the candidate genes real time-PCR method were compared with microarray data Gene Expression Omnibus (GEO accession #GSE4694) (13).

Statistical analysis:

The data are presented as mean GS.E.M. To determine statistical significance, Student's t-test. was applied. If not indicated otherwise the criterion for significance was set at ($P \leq 0.05$). All cell culture and real time experiments were performed in triplicate.

RESULTS

Characterization of C2C12 differentiation:

Myoblast cells have proliferated in the presence of serum and differentiated upon partial serum deprivation from myoblast cells caused their differentiation into myocytes, confirmed by qRT-PCR result for specific

skeletal marker, myoD .C2C12 myoblast type have confirmed by up regulation, for precursor cells marker, Pax7 (figure 1).

Bioinformatically predicted targets

In order to bioinformatics prediction , we found that miR-135 targeted several signal molecules, regulating myogenesis process such as adiponectin receptor 2 (ADIPOR2), STX4-binding protein (STXBP4), Rho-associated coiled-coil containing protein kinase 1 (ROCK1) and vesicle-associated membrane protein 2 (VAMP2) .

Changes in predicted targets expression during C2C12 differentiation

We have examined the expression level of predicted targets , in mouse C2C12 cells during differentiation,. Interestingly according to qRT-PCR analysis, it had been shown that expression level of ADIPOR2 and VAMP2 were up-regulated in differentiated cells while ROCK1 and STXBP4 had shown decreased pattern .

Evaluation of real time RT-PCR result by microarray data

To extend the results of our real time RT-PCR analysis, we analyzed all transcripts by an available microarray data (13). The raw data of microarray signal values have gotten form (GEO accession #GSE4694) . Chen et al. (2006) had analyzed three different RNA samples from proliferating and from differentiated C2C12 cells , individually (6 microarrays total).

For two of the transcripts examined, ADIPOR2 and VAMP2 microarray results have reflected the same up-regulation (p-value ≤ 0.05) and for STXBP4, microarray result has reflected the same down-regulation (p-value ≤ 0.05) trends as revealed by the Real time RT-PCR data (figure 3). However, the magnitude of microarray data changes was different from real time RT-PCR result. The microarray result of ROCK-1 was not suitable for this comparison because of noises.

DISCUSSION

Follow a line of investigation on growth mechanisms of skeletal muscle is an important duty in animal science and human medical research (14-15).We had previously identified that miR-135 expression was up-regulated during myogenic differentiation and may be involved in skeletal muscle development (10). According to our previous results, the aim of this study was to investigate the expression changes of bioinformatically selected miR-135 targets to better understand the molecular mechanism of muscle differentiation process.

We found that expression level of ADIPOR2 and VAMP2, were up-regulated in differentiated cells Furthermore, our qRT-PCR analysis showed that ROCK1, and STXBP4 were already expressed in proliferating C2C12 cells, but its level was significantly down-regulated following the induction of differentiation (figure2) (p-value $\leq$ 0.05) .

ADIPOR2, have been identified to function as adiponectin receptor in human and mouse skeletal muscle (16). Activation of adiponectin /ADIPOR2 was shown to stimulate protein synthesis through insulin signaling , PI3K/Akt and rapamycin (mTOR) pathway. On the other hand, AMP-activated protein kinase (AMPK) was reported to interfere with the mTOR pathway. (17-20). Besides mTOR and PKB, other kinases known to be involved in signaling pathways of cellular growth such as p38 mitogen-activated protein kinase (p38 MAPK), or p44/42 mitogen-activated protein kinase (p44/42 MAPK; ERK 1/2)(19-20). All these molecules could be activated through ADIPOR signaling cascade and participated in myogenesis.

Activation of ADIPOR2 stimulate both AMPK and PI3K/Akt pathway (16, 21-23). Therefore up regulation of ADIPOR2 may accelerate myogenesis, because of increasing PI3K/Akt/mTOR pathway.

Base on our result , in C2C12 cell line, VAMP2 was expressed at a low level in the proliferating stage, but increased after differentiation. VAMP2 is an important member of SNARE proteins, which regulate the docking and fusion of vesicles with their target membrane (24) play roles in construction and maintenance of membrane structures (25). In addition, some muscular disorders result from mutations in genes occupied in membrane traffic, e.g., dysferlinopathy and caveolinopathy (26), indicating the importance of membrane traffic in muscle cells. Active membrane trafficking is considered to play a role in the growth and continuance of skeletal muscle (25). VAMP2 has a well-established role in the sorting of membrane proteins .Moreover, the fusion of myoblasts is regulated by membrane proteins. Thus, up regulation of VAMP2 as one of the key component in Membrane traffic, could participate in myogenic differentiation via the maintenance and development of myofibers.

Using Affymetrix cDNA microarray (GEO accession #GSE4694 done by Chen et.al) analysis to compare the transcript levels ADIPOR2 and VAMP2 in undifferentiated C2C12 cells vs. differentiated populations confirmed

our data but not for STXBP4 (p-value > 0.05). According to our study, RT-PCR revealed that the levels of ADIPOR2 and VAMP2 transcripts more increased in comparison with microarray analysis fold changes (figure 2). It is obviously reasonable, because real time RT-PCR technique provides a more accurate representation of changes in the level of specific transcripts in the differentiated vs. undifferentiated C2C12 cells than the microarray analysis, since the real time PCR signal is linear over a wide concentration range (13,27), as confirmed by serial dilution experiments with different samples (data not shown).

It appears that miR-135 participates in myocyte formation process through targeting components (perhaps inhibitors of muscle growth) of myogenesis except for ADIPOR2 and VAMP2 because both miRNA and these two predicted target have increased expression pattern during differentiation. It seems that, ADIPOR2 promotes myoblast proliferation in cooperation with mTOR. Furthermore, VAMP2 is associated with insulin signaling, and Active membrane trafficking pathway which lies at the regulatory network controlling muscle development. For that reason, increasing expression of both genes could be necessary during myogenic differentiation. Indeed, it might be the result of increasing mTOR activation and maintenance and development of muscle cells.

Decreased expression of ROCK1 and STXBP4 have been shown in C2C12 myocyte in comparison with primary muscle cells in myogenic differentiation.

STXBP4 plays a role in the translocation of transport vesicles from the cytoplasm to the plasma membrane in muscle cells. STXBP4 inhibits the translocation of intracellular vesicles to the plasma membrane by syntaxin binding and preventing the interaction between syntaxin and VAMP2 (28-29). Thus down regulation of STXBP4 seems to enhance cellular fusion. Decreased expression of STXBP4 accompanied with overexpression of miR-135 (as its possible target) could be involved in the control the vesicle translocation which is required for maintenance and development of cells during differentiation.

Recent studies have demonstrated that ROCK1 acts independently of RhoA to prevent cell fusion and myotube formation (30-31) and Conversely, the inhibition of via siRNA leads to accelerated progression in cell myoblast fusion (32-33). According to our assay, down regulation of ROCK1 (potential target of miR-135) as a negative regulator of myogenesis accompanied with overexertion of miR-135. Hence, it sounds as if miR-135 could be involved in differentiation via targeting ROCK-1 and lead to myogenesis.

All together, it seems that miR-135 could be involved in myogenesis via down regulation of STXBP4 and ROCK-1 except for ADIPOR2 and VAMP2 which their over expression is essential for muscle cell differentiation.

CONCLUSION

Further studies will be needed to concentrate on the roles of these genes and miR-135. Determination of myogenesis molecular pathways , will enhance our outlook of muscle development mechanism. Using informatics, biochemical and genetic approaches together , will help us to establish new therapeutic approaches by identifying functional gene candidates as potential targets for clinical purpose.

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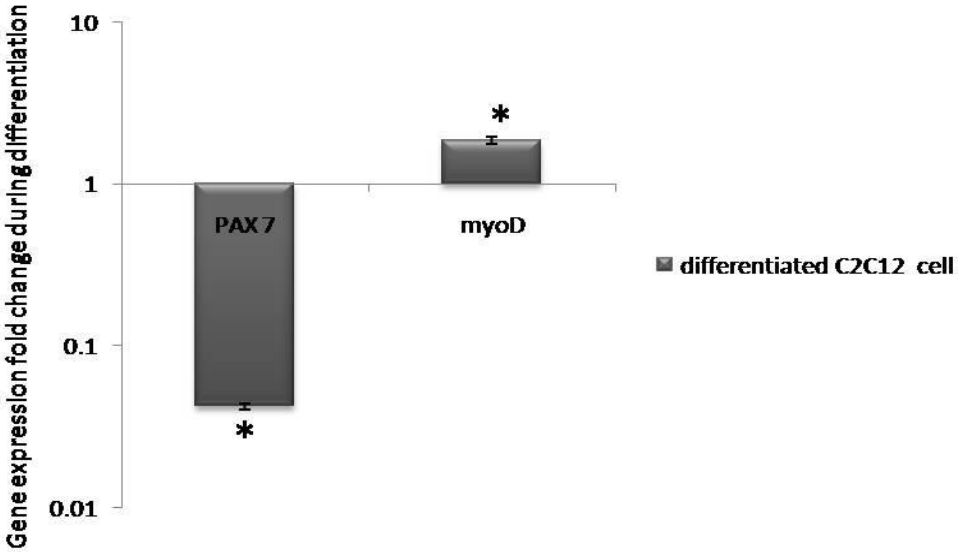

Fig 1: After that myoblasts were induced to differentiate with DMEM medium containing 3% horse serum for 3 days, differentiation process had confirmed by down-regulated profile of PAX7(myoblast marker) and overexpresion of myoD (myocyte marker) (p-value≤0.05) . Error bars indicate SEM (n=3)

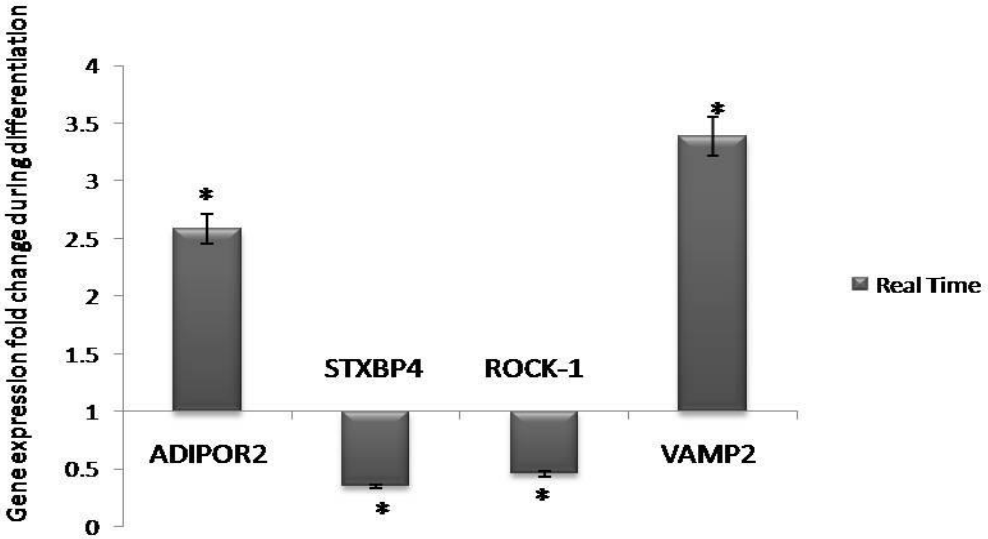

Fig 2: Expression pattern of candidate genes during myogenesis. Based on real time results concurrent to ,miR-135 up-regulation in differentiated cells, STXBP4and ROCK-1 has significant down-regulated profile during differentiation process (p-value≤0.05). In contrast, overexpresion of ADIPOR2 and VAMP2 gene expression were observed significantly (p-value >0.05). Error bars indicate SEM (n=3)

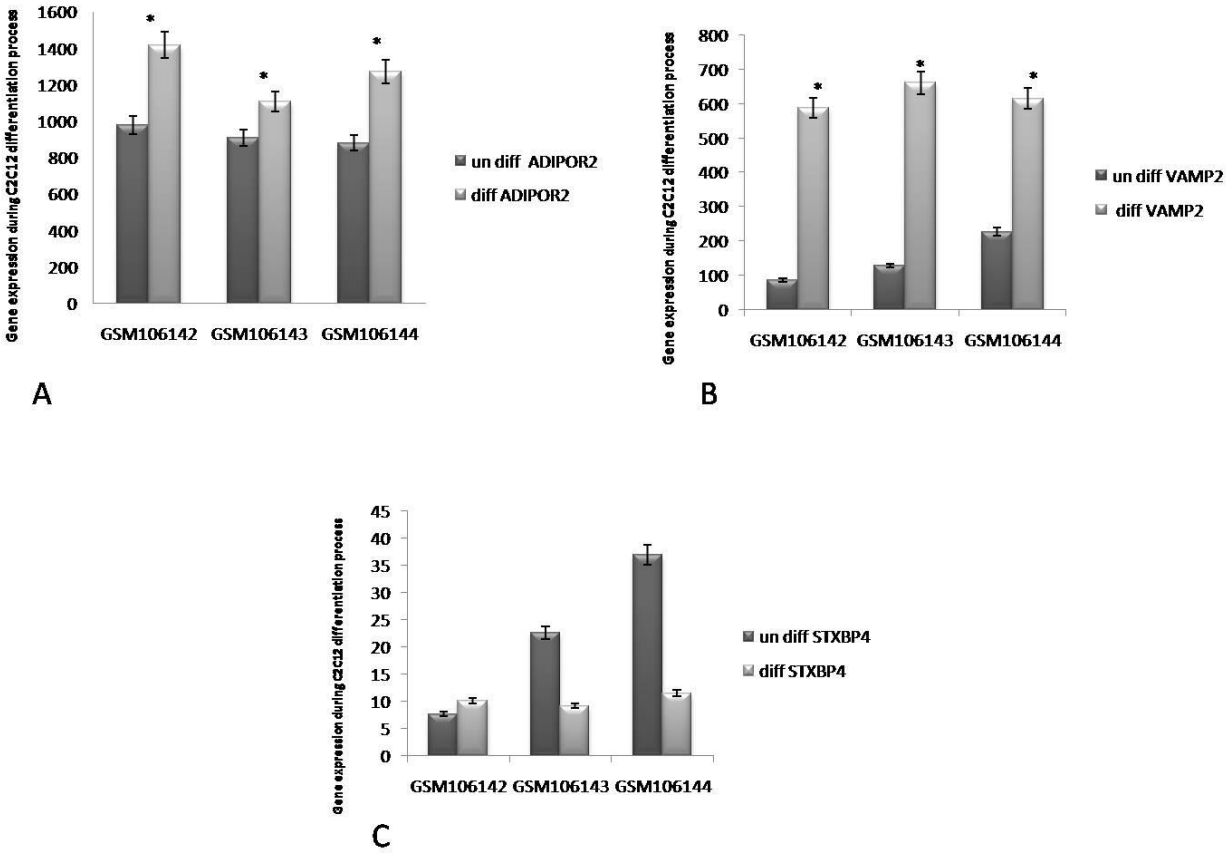

Fig3; miR-135 predicted targets (ADIPOR2,VAMP2 and STXBP4) expression levels during myogenesis using microarray data GEO accession #GSE4694 . The expression changes in all microarray outcomes are the same as our qReal time PCR. In all tree samples and Muscle micro array for VAMP2 and ADIPOR2 were valuable (p-value≤0.05) (A-B) except for STXBP4 result (p-value >0.05) (C). Error bars indicate SEM (n=3)

Table1: Gene-specific primers designed for Real time PCR assay

Primer	Sequence
Real time PCR	
ADIPOR2	Forward : 5` AATTGGATTACTCTGGTATTGC 3`
	Reverse : 5` CACGAACACTCCTGCTCTG 3`
STXBP4	Forward : 5` TTGATATGGACTGCTTACCTTAC 3`
	Reverse :5` TCCTCTTCACTCTCCTCTGC 3`
ROCK1	Forward:5` TTGTTTGCTGGATGGATTG 3`
	Reverse:5` CTCTGCCGATTACCTTTACC 3`

VAMP2	Forward:5`GTCACTGCCTCTGCCAAG3`
	Reverse:5`GTCCACCACCTCATCCAC3`

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